Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1758 (2006) 1732–1742
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.elsevier.com/locate/bbamem

Membrane interaction and activity of the glycolipid transfer protein

Gun West [1], Matts Nylund [1], J. Peter Slotte, Peter Mattjus *

Department of Biochemistry and Pharmacy, Åbo Akademi University, Tykistökatu 6A, 3rd floor, FI-20520, Turku, Finland

Received 25 February 2006; received in revised form 29 May 2006; accepted 14 June 2006
Available online 7 July 2006

Abstract

In this study we have addressed the ability of the glycolipid transfer protein (GLTP) to transfer anthrylvinyl-galactosylceramide at different pH and sodium chloride concentrations, and the ability of three different mutants to transfer the fluorescently labeled galactosylceramide between donor and acceptor model membranes. We constructed single tryptophan mutants with site-directed mutagenesis where two of the three tryptophan (W) of wild-type human GLTP were substituted with phenylalanine (F) and named W85 GLTP (W96F and W142F), W96 GLTP (W85F and W142F) and W142 GLTP (W85F and W96F) accordingly. Wild-type GLTP and W96 GLTP were both able to transfer anthrylvinyl-galactosylceramide, but the two variants W85 GLTP and W142 GLTP did not show any glycolipid transfer activity, indicating that the tryptophan in position 96 is crucial for transfer activity. Tryptophan fluorescence emission showed a blue shift of the maximal emission wavelength upon interaction of glycolipid containing vesicle with wild-type GLTP and W96 GLTP, while no blue shift was recorded for the protein variants W85 GLTP and W142 GLTP. The quantum yield of tryptophan emission was highest for the W96 GLTP protein whereas W85 GLTP, W142 GLTP and wild-type GLTP showed a lower and almost similar quantum yield. The lifetime and anisotropy decay of the different tryptophan mutants also changed upon binding to vesicles containing galactosylceramide. Again wild-type GLTP and W96 GLTP showed similar behavior in the presence of vesicles containing glycolipids. Taken together, our data show that the W96 is involved not only in the activity of the protein but also in the interaction between the protein and glycolipid containing membranes.

Keywords: Fluorescence; Site-directed mutagenesis; GLTP; Membrane; Binding

Proteins that specifically bind and transfer glycolipids between artificial membranes, termed glycolipid transfer proteins (GLTP), have been identified in many cell types [1] and are emerging as an interesting family with a unique structural fold [2,3]. A similar type of protein has also been identified in the filamentous fungi *Podospora anserina,* where a gene product called HET-C2 shows glycosphingolipid transfer activity and GLTP-like characteristics [4]. Another protein, accelerated cell death protein 11 (ACD11) identified in cells from *Arabidopsis thaliana* show *in vitro* sphingosine transfer activity but no transfer of glycolipids, and only low GLTP sequence similarity [5]. Database searches also reveal a protein homologous to mammalian GLTP, phosphoinositol 4-phosphate adaptor protein-2 (FAPP2, gene accession number Q96JA3), which contain a GLTP domain and a pleckstrin homology domain. FAPP2 appears to localize to the trans-Golgi network on nascent carriers, and interact with PtdIns[4]P and the small GTPase ADP-ribosylation factor (ARF) through their PH domain [6].

The preliminary structure analysis of native human GLTP using steady-state fluorescence approaches was done recently [7]. The study suggested that 75% of the average tryptophan (W) fluorescence was accessible to soluble quenching agents. Also the total tryptophan fluorescence responds to conformational changes induced by ionic strength and the presence of glycolipid liposomes [7]. The crystallization of bovine GLTP was done recently showing that the crystals belong to primitive space group $P2_1$ [8]. The complete 3D

Abbreviations: GLTP, glycolipid transfer protein; DiO-C_{16}, dihexadecylo-xacarbocyanine perchlorate; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-choline; GalCer, galactosylceramide; AV-GalCer, N-[(11*E*)-12-(9-anthryl)-11-dodecenoyl]-1-O-β-galactosylsphingosine; NATA, N-acetyltryptophanamide

* Corresponding author. Tel.: +358 2 2154271; fax: +358 2 2154745.
E-mail address: Peter.Mattjus@abo.fi (P. Mattjus).

[1] Both authors contributed equally to this work.

0005-2736/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2006.06.020

structure of bovine GLTP was recently solved [2]. The bovine GLTP with the bound glycosphingolipid resembles human GLTP that folds with a previously unknown two-layer all-α-helical topology [3]. The glycosphingolipid is anchored with the sugar-amide head group to the GLTP recognition center by hydrogen bonding networks and hydrophobic contacts. Both acyl chains are encapsulated in a hydrophobic cavity [2,3]. How GLTP interacts with membranes and what part of the protein that seems to recognize membrane interfaces is still unclear, as well as potential structural changes taking place during the substrate recognition and binding events.

To gain further insight into the dynamic structure and membrane interaction of GLTP we have produced three different mutant analogues of human GLTP. Changes in tryptophan fluorescence emission intensity are commonly observed when peripheral proteins interact with membranes [9]. Often the emission intensity increases and the emission maximum undergoes a blue shift when a protein with tryptophan moves from an aqueous milieu and becomes membrane-associated. Two of the three tryptophan of wild-type GLTP were substituted with phenylalanine (F) using site-directed mutagenesis and named according to the remaining tryptophan, W85 (W96F and W142F mutated), W96 (W85F and W142F mutated) and W142 (W85F and W96F mutated). A wide range of experimental approaches was used to examine the GLTP-vesicle interaction process, including fluorescence quenching, circular dichroism spectroscopy, steady-state and lifetime fluorescence analysis, and lipid transfer activity measurements. It was found that the conservative replacement of tryptophan in position 96 with phenylalanine resulted in a loss of transfer activity. Keeping W96 but replacing tryptophan 85 or 142 with phenylalanine gave an active protein. Wild-type GLTP and W96 GLTP adsorption to phospholipid vesicles with or without glycolipids caused a blue shift in the tryptophan emission suggesting that the microenvironment around W96 is involved in the process where GLTP is scooting for glycolipids in membranes. This blue shift was not observed with the GLTP mutants with W85 and phenylalanine in position 96 and 142, or with GLTP mutant with W142 and phenylalanine in position 85 and 96. We conclude that W96 is crucial for transfer activity, and additionally both W85 and W142 are needed for a fully functional protein.

1. Materials and methods

1.1. Reagents

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and palmitoyl-GalCer (GalCer) was purchased from Avanti Polar Lipids (Alabaster, AL), and the phospholipid concentration was determined by the method of Bartlett [10], and for galactosylceramide gravimetrically. The fluorescent probe, N-[(11*E*)-12-(9-anthryl)-11-dodecenoyl]-1-O-β-galactosylsphingosine (AV-GalCer) was prepared as described earlier [11]. 3,3′-Dihexadecyloxacarbocyanine perchlorate (DiO-C_{16}) was from Molecular Probes (Eugene, OR) and Triton X-100 was from ICN Biomedicals (Aurora, OH). Acrylamide (Invitrogen, Carlsbad, CA), guanidine hydrochloride (Fluka, Steinheim Germany) and N-acetyltryptophanamide, NATA (Sigma-Aldrich, St. Louis, MO) were all high-purity grade.

1.2. Expression and purification of wild-type GLTP and single tryptophan mutants

Tryptophan (TTG) was replaced to phenylalanine (TTT) using a QuickChange site-directed mutagenesis kit from Strategene. Human GLTP gene (NM_016433) cloned into a pGEX-6P-vector (Amersham Biotech) was used as a template. The following oligonucleotides were used for the mutations; 5′-GAAATGTATGGAGCAGAG**TTT**CCTAAAGTAGGGGCCACACTG-3′, 5′-GCCACACTGGC GCTGATG**TTT**CTTAAAAGAGGCCTCCGC-3′ and 5′-GCCCTTAAGAAGTACCATGGC**TTT** ATCGTGCAGAAG ATCTTC-3′.

The vectors of wild-type and mutants were transformed into *Escherichia coli* BL21 cells and grown in yeast-tryptone medium at 29 °C until cell density A_{600} reached 0.6. Expression of the glutatione-S-transferase fusion protein construct was induced with isopropyl-1-thio-β-D-galactopyranoside at a final concentration of 1 mM. After 2 h of incubation the cells were harvested and lysed by incubation with lyzosyme in PBS pH 7.0 followed by sonication. The cleared lysate was purified on a column packed with Glutathione Sepharose 4B beads (Pharmacia Biotech, Uppsala, Sweden). Elution of the protein was done with PreScission Protease in 10 mM sodium dihydrogen phosphate buffer pH 7.0. The purity was confirmed by analysis on a SDS-PAGE gel (16%) and Coomassie staining. The yield for wild type GLTP and all mutants was between 1.8 and 2.8 mg protein per litre culture media.

1.3. Glycolipid transfer assay

The resonance energy transfer (RET) assay for measuring AV-GalCer transfer has been thoroughly described recently [12] and is based on the method described earlier [13]. For determining the transfer rate at varying pH a sodium phosphate buffer containing 10 mM sodium dihydrogen phosphate, 1 mM dithiothreitol and 1 mM EDTA with the respective pH (4.0, 5.5, 7.0, 8.5 and 10.0) was used both in the preparation of the respective vesicles and as measuring solution. The pH of the buffer did not change during the transfer reaction, and the pH was check both before and after the measurements were done. For the measurement of the transfer activity at different NaCl concentrations the same sodium dihydrogen phosphate buffer at pH 7.0 with varying concentrations of NaCl was used. The vesicles were prepared by probe sonication. POPC with 1 mol% AV-GalCer and 3 mol% DiO-C_{16} (non-transferable quencher) was dried under nitrogen and dissolved in the buffer. The suspension with a total lipid concentration of 0.4 mM was sonicated for 10 min, on ice, with a Branson 250 sonifier, and then centrifuged for 15 min at 15000 rcf to remove titanium probe particles, multilamellar vesicles, and undispersed lipid (negligible amount) [14]. The final concentration of the donor vesicles per assay was 13 μM, the final AV-GalCer concentration in each assay was 0.13 μM. The acceptor vesicles (final concentration 130 μM) were probe sonicated POPC vesicles in a ten-fold excess compared to the donor vesicles. The protein concentration was 28 nM per assay.

1.4. Circular dichroism measurements

Far-UV spectra were measured on a Jasco 720 spectropolarimeter (Tokyo, Japan) with a scanning speed of 100 nm/min. The measurements were done with a 1 mm path length cuvette and a protein concentration of 9.5 μM made up in 10 mM phosphate buffer pH 7.0. The temperature was set at 37 °C and the spectra were recorded in a range of 185–260 nm with 1 nm bandwidth. All the recorded spectra are averages of 10 scans.

1.5. Acrylamide quenching experiments

Quenching experiments were carried out on a Cary Eclipse spectrofluorometer (Varian, Palo Alto, CA), equipped with a temperature controlled cuvette holder with stirring. The samples were excited at 295 nm and fluorescence measured in a range of 310–450 nm with both emission and excitation bandwidths on 10 nm. The temperature was maintained at 37 °C throughout the measurements. The samples were denatured by incubation with 6 M guanidine hydrochloride for 2 h at 25 °C. Aliquots of the acryl amide stock quencher solutions (6 M) made up in buffer (10 mM sodium dihydrogen phosphate pH 7.0) were directly added to the sample under rapid stirring and

allowed to mix for 2 min before measuring. The protein concentration was kept at $OD_{295}<0.1$ to avoid inner filter effects. Data were corrected for dilution effects and analyzed using the Stern–Volmer equation:

$$\frac{F_0}{F} = K_{SV}[Q], \tag{1}$$

where F_0 and F are the fluorescence intensity in the absence and presence of quencher. $[Q]$ is the concentration of the quencher, K_{SV} is the Stern–Volmer quenching constant. In a protein, which contains several tryptophan residues, the presence of different classes of tryptophan residues, exposed or buried, is reflected in the Stern–Volmer plot [15]. The fraction of the total fluorescence accessible to the quencher can be calculated from the modified Stern–Volmer plot (Lehrer plot [16])

$$\frac{F_0}{\Delta F} = \frac{1}{K_Q[Q]} + \frac{1}{f_a}, \tag{2}$$

where F_0 and $[Q]$ are same as defined earlier and ΔF is the change in the fluorescence intensity due to quenching, K_Q is the Stern–Volmer quenching constant of the exposed tryptophan residues and f_a is the fraction of the initial fluorescence, which is accessible to the quencher.

1.6. Fluorescence titration measurements

Interactions between GLTP and membranes were studied by monitoring the changes in the tryptophan fluorescence emission spectra of the GLTP variants upon addition of probe sonicated small unilamellar vesicles (25–40 nm in diameter). Fluorescence of wild-type GLTP and the individual single tryptophan mutants was measured before and after addition of different amounts of POPC vesicles or POPC with 10 mol% GalCer to a 4.2 μM protein solution. Tryptophan fluorescence (295 nm excitation) was measured between 310 and 420 nm at 37 °C on a Cary Eclipse spectrofluorometer with excitation bandwidth at 5 nm and emission bandwidth at 5 nm. The emission intensities are corrected for the increasing light scattering of the added vesicles.

1.7. Quantum yield measurements

Quantum yield measurements were performed on a PTI QuantaMaster 1 (Photon Technology International, New Jersey, NJ) spectrophotometer operating in the T-format and equipped with a temperature controlled cuvette holder with stirring. Excitation was set to 295 nm (bandwidth 6 nm) and emission measured between 310 nm and 450 nm with a bandwidth of 6 nm. A cross-oriented configuration of the polarizers Ex_{pol} 90° and Em_{pol} 0° provided maximal suppression of liposome-scattering artefacts, as well as a short emission path [9]. The cuvette was orientated to have a longer (10 mm) path length of the excitation than for the emission (1 mm) [9]. In order to avoid inner filter effects the optical density OD_{295} was kept below 0.05 in all measurements. All fluorescence spectra were corrected for the buffer and the wavelength dependence of the emission monochromator and the photomultiplier. The quantum yields were determined relative to N-acetyltryptophanamide (NATA) in aqueous solution according to the method of Parker and Rees [17] using a quantum yield for N-acetyltryptophanamide in water of 0.177 at 37 °C

$$Q_{prot} = \frac{\int I_{prot}}{\int I_{NATA}} \frac{A_{NATA}}{A_{prot}} Q_{NATA}, \tag{3}$$

were I is the integrated intensity of the wavelength region 310–450 nm, Q is the quantum yield, A is the absorbance at 295 nm.

1.8. Time-resolved fluorescence measurements

The system used a GL-330 pulsed nitrogen laser as a source of excitation (Photon Technology International). The nitrogen laser produced a train of 500-ps excitation pulses at 337 nm at a repetition rate of 10 Hz. The dye laser output at 590 nm was frequency-doubled to 295 nm with a GL-103 frequency doubler coupled to an MP-1 sample compartment via fiber optics. Rhodamine 6G chloride (Invitrogen, Carlsbad, CA) was used as the laser dye. Lifetime emission profiles were collected at the magic angle (54.7°) of emission polarizer to avoid any contribution from anisotropy. The instrument response functions were obtained from a scattering solution of glycogen. The apparatus response was always checked by measuring the lifetime of NATA in water as a standard (3.17 ns) before starting the accumulation of data for the protein samples.

Anisotropy decays were collected by measuring a repeated cycle of the parallel ($I_{||}$) and perpendicular ($I_{\perp}$) fluorescence intensity components. It was found that 10 cycles were sufficient to give a good signal-to-noise ratio. Fluorescence decays were analyzed with the Felix 32 analysis package using a discrete one- to four-exponential fitting program. The weighted-average lifetimes were calculated from the results of multiexponential fits by using the expression

$$\langle \tau \rangle = \frac{\Sigma \alpha_i \tau_i^2}{\Sigma \alpha_i \tau_i}, \tag{4}$$

where a_i and τ_i represented pre-exponential factors and lifetimes, respectively. The rotational correlation time ϕ was calculated from the deconvoluted anisotropy fit curves using the software supplied by the spectrofluorometer manufacturer.

2. Results

2.1. Protein expression

Human GLTP contains three tryptophan (W85, W96 and W142). To examine the environment of the individual tryptophan more closely, single tryptophan mutants were constructed, as described in Materials and methods. Briefly, with site-directed mutagenesis two of the tryptophan were replaced with phenylalanine (W→F) and the mutants were named after the remaining tryptophan. The mutations were confirmed with sequencing (data not shown). The protein constructs were expressed as described in Materials and methods. The purity was 95% judged by SDS-PAGE (data not shown).

2.2. Glycolipid transfer activity analysis

The activity of wild-type GLTP and the three single tryptophan mutants are shown in Fig. 1. The transfer rate of AV-GalCer between small unilamellar vesicles (POPC) of wild-type GLTP was normalized to 1. The initial transfer rates are calculated after the first minute after GLTP addition. The mutant W96 GLTP (with the W96 intact but with tryptophan at 85 and 142 replaced by phenylalanine) showed only a slightly decreased transfer rate (72% of wild-type GLTP), whereas both W85 GLTP and W142 GLTP showed no significant transfer rate (<6% of wild-type GLTP, Fig. 1). In a recent study it was shown that a point mutation of W96 to phenylalanine maintained 63% of its activity, and a mutation of W96 to alanine resulted in a inactive protein [3].

Previously we have analysed membrane charged conditions for effects in transfer activity [18,19]. It is likely that GLTP in biological systems will encounter different environments with varying salt and pH conditions. Therefore the sensitivity to variations in the pH and NaCl concentration for the wild-type GLTP and the mutants were examined. The transfer activity of WT GLTP and W96 GLTP related to varying pH values were analysed at five different values between pH 4 and pH 10 (Fig. 2). Since the mutants W85 GLTP and W142 GLTP did

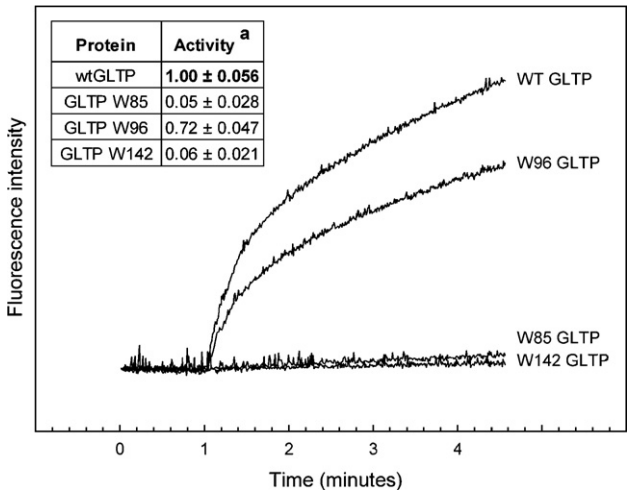

Fig. 1. Anthrylvinyl-galactosylceramide (1 mol%) transfer activity of wild-type GLTP (WT GLTP) and the three different single tryptophan GLTP mutants. (a) The transfer was measured between probe sonicated small unilamellar POPC donor and acceptor vesicles (in a ten-fold excess, 25-40 nm in diameter) at 37°C using 28 nM protein per assay after the first minute of GLTP addition. The graphs shows the transfer activity of the different GLTP variants at 37 °C. The activities were normalized to the rate for wild-type GLTP, and are averages of at least 3 different measurements.

not show any transfer activity, they were not analyzed. The highest activity was found at pH 7.0 for WT GLTP, with much lower values at pH 4.0, only 11% and at pH 10.0 only 66% compared to pH 7.0 (Fig. 2A). This clearly suggests that GLTP is more sensitive to acidic conditions. The W96 GLTP showed a slightly higher activity at pH 8.5 than at 7.0, but was also sensitive to acidic conditions (Fig. 2B). In Fig. 2C the initial transfer rate versus pH is plotted to illustrate the dependence of pH on the transfer activity for the two active proteins.

The transfer activity of wild-type GLTP and W96 GLTP in a sodium phosphate buffer (10 mM) pH 7.0 with increasing concentration of NaCl from 0 to 200 mM is presented in Fig. 3. The optimal concentration was found to be between 150 and 200 mM NaCl for both wild-type GLTP (Fig. 3A) and W96 GLTP (Fig. 3B) with a significantly less transfer activity for 100 mM and lower concentrations also for both proteins. The dependence of NaCl concentration on the initial transfer rate for WT and W96 GLTP is presented in Fig. 3C.

2.3. Far-UV circular dichroism analysis

Circular dichroism is a useful technique to analyze structural changes in protein secondary structure. To determine if the observed lack of activity for the W85 GLTP and the W142 GLTP mutants was due to secondary structural changes, a far-UV CD spectra for wild-type GLTP and each mutant was recorded (Fig. 4). The spectra of the mutants were similar to that of wild-type GLTP with a minimum around 208 nm and 221 nm typical of α-helices. The CD data were further analysed using the CDPro software [20,21]. The software analysis detected no apparent differences in the secondary structure between the wild-type GLTP and the three tryptophan mutants (data not shown). In addition, the presence of POPC or POPC vesicles containing 1 mol% GalCer produced no apparent changes in the CD spectra (Fig. 4).

2.4. Tryptophan fluorescence and quantum yield

The emission maximum wavelength of tryptophan is characteristic for the polarity of its microenvironment. To

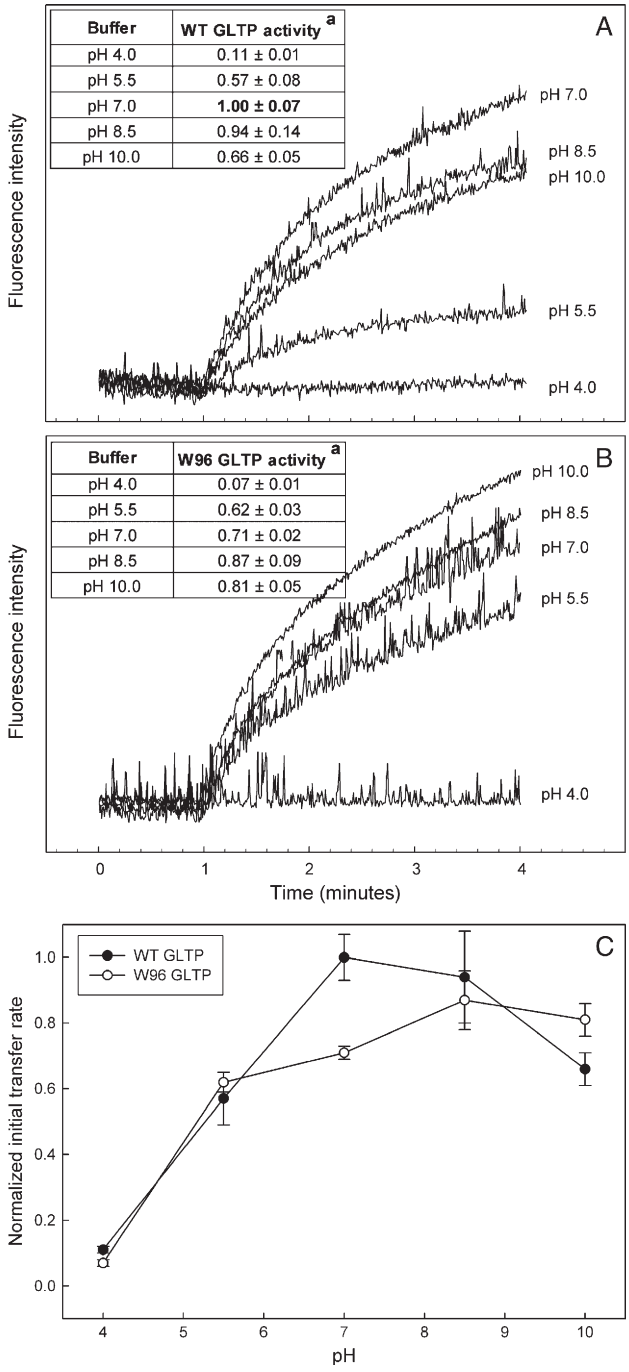

Fig. 2. Representative transfer activity curves for pH dependent activity of (A) wildtype GLTP and (B) W96 GLTP. (a) The transfer of AV-GalCer was measured between small unilamellar POPC donor and acceptor vesicles at 37°C using 28 nM protein per assay at 37 °C. The relative transfer rate is expressed as 1 at pH 7.0 for wild-type GLTP. (C) Represents the relative transfer rates versus pH for WT GLTP and W96 GLTP. Averages are of at least 3 different measurements.

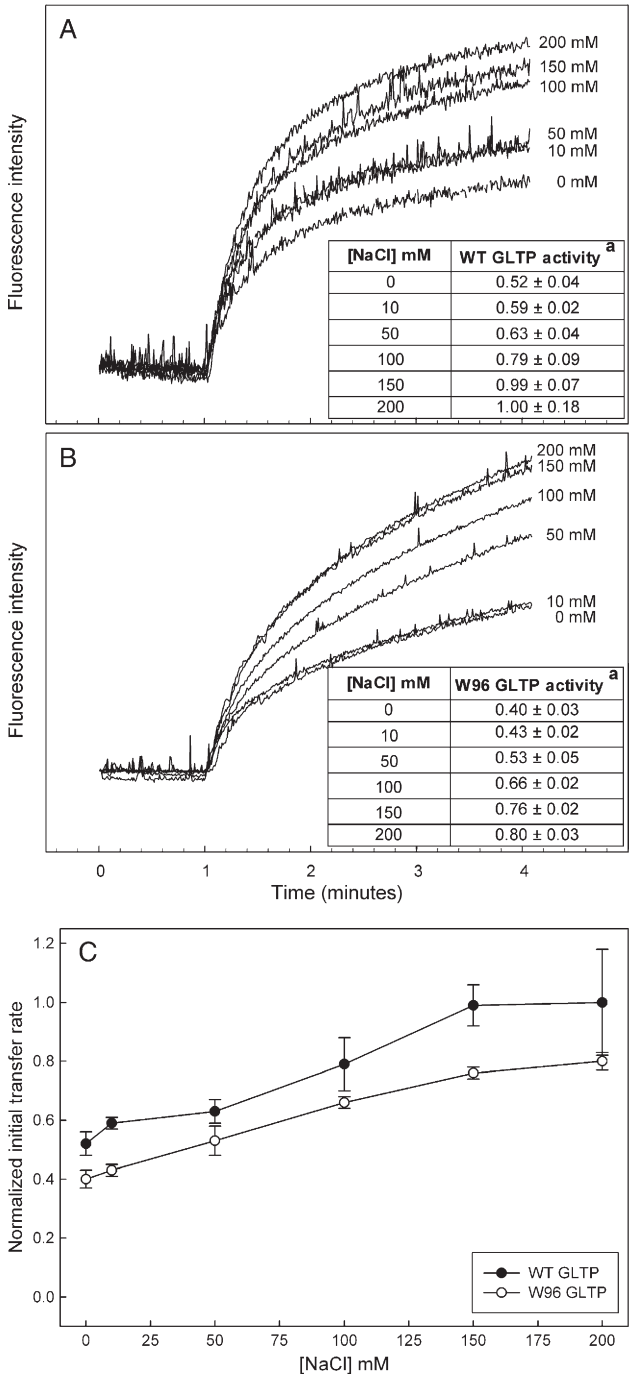

Fig. 3. Representative transfer activity curves for NaCl dependent activity of (A) wildtype GLTP and (B) W96 GLTP. The transfer of AV-GalCer was measured between small unilamellar POPC donor and acceptor vesicles at 37°C and pH 7.0 using 2 μg protein per assay. (a) Protein (28 nM) mediated AV-GalCer transfer from and to POPC vesicles in a 10 mM sodium dihydrogen phosphate buffer pH 7.0 at various NaCl concentrations at 37 °C. The relative transfer rate is expressed as 1 at [NaCl] 200 mM. (C) Shows the relative transfer rates versus the NaCl concentration for WT GLTP and W96 GLTP. Averages are of at least 3 different measurements.

selectively excite only tryptophan, a wavelength of 295 nm was used to determine the tryptophan fluorescence emission maxima of the protein constructs. Wild-type GLTP showed a fluorescence emission maximum at 348 nm (Table 1) indicating an average polar environment for the indole side chains. The maximum wavelength for the mutant W85 GLTP and W142 GLTP was 351 nm and 349 nm respectively, and the W96 GLTP mutant was blue shifted 3 nm with a maximum of 345 nm. After incubation of the different proteins with guanidine hydrochloride for 2 h the maximum wavelength shifted to 350–352 nm in all GLTP variants, indicating water exposure of all tryptophan residues due to unfolding, Fig. 5.

Tryptophan quantum yield in proteins is highly unpredictable, and usually lie anywhere between 0.01 and 0.3. A reduced quantum yield in protein is usually reflected by electron transfer from the excited tryptophan to acceptor sites, or if tryptophan molecules participate in hydrogen bonding networks [22]. The wild-type GLTP and mutants W85 GLTP and W142 GLTP have similar quantum yield values while mutant W96 GLTP possess a higher value (Table 1). This could indicate that the tryptophan in W96 GLTP is located in a more non-polar environment than in the W85 GLTP and W142 GLTP variants.

2.5. Fluorescence titration

To examine whether emission from different tryptophan would be sensitive to lipid membranes we performed a titration experiment with POPC or POPC containing 10 mol% palmitoyl-GalCer vesicles, added to the GLTP constructs. In Fig. 6 we show that the emission of tryptophan blue shift only for the WT GLTP and W96 GLTP (Fig. 6A and C) protein when POPC vesicles are added, where as W85 GLTP and W142 GLTP does not blue shift (Fig. 6B and D). The same pattern is seen if POPC vesicles containing 10 mol% GalCer is added with the difference that the shift is more pronounced (Fig. 6). If the polarity of the tryptophan environment changes as a result of adsorption to the membrane interface, a shift in the emission wavelength would be expected. No change in tryptophan emission properties would be an indication that the tryptophan environment is not affected by membrane interface. In all measurements the fluorescence excitation was set to 295 nm in order to avoid fluorescence from GLTPs 10 tyrosine residues and the emission maxima was plotted as a function of lipid concentration. Wild-type GLTP and W96 GLTP binding to vesicles without GalCer (Fig. 7A and C, filled circles) was linearly decreasing as a function of lipid concentration, while W85 GLTP and W142 did not show any change in tryptophan emission blue shift (Fig. 7B and D). Wild-type GLTP and W96 GLTP binding to GalCer containing POPC vesicles on the other hand showed a abrupt decrease in the tryptophan fluorescence emission intensity (up to 0.14 mM POPC vesicles), followed by a slower decrease at more saturating conditions (Fig. 7A and C, open circles).

2.6. Tryptophan quenching by acrylamide

Tryptophan residues deeply buried in a protein are generally blue shifted, with an emission maximum near 330 nm, and are inaccessible to quenching by acrylamide [23], Fig. 5. Tryptophan fluorescence measurements and acrylamide quenching were used to obtain information about the individual single tryptophan fluorescence. Acrylamide is often used since it is a

polar, uncharged molecule that quenches the fluorescence of indole derivatives exposed to the aqueous milieu. Addition of 0.28 M acrylamide to native and denatured wild-type GLTP and the three different mutants resulted in linear curves in the Stern–Volmer plots (data not shown) indicating that the quenching is dynamic (Table 2). The Stern–Volmer constant, K_{SV}, is derived from the slope of the curve. The obtained Stern–Volmer constants for wild-type GLTP and the mutants are higher for the denatured protein than for the native protein. Unexpectedly, the value of native W142 GLTP is low (K_{SV}=3.63) indicating a relatively hydrophobic surrounding. K_{SV} values can by themselves be misleading [24]. Because of this the modified Stern–Volmer plot was used to calculate the accessible fraction, f_a, which is more suitable in comparing different protein constructs. This shows that mutants W85 GLTP and W142 GLTP are fully accessible to the quencher while W96 GLTP has a fraction not accessible to acrylamide. The intrinsic average tryptophan fluorescence in wild-type GLTP was quenched to 69% and this would indicate that the inaccessible fraction could come from the W96, explaining the data obtained previously by Li and co-workers [7].

Table 1
Emission maxima and quantum yield (with respect to N-acetyltryptophanamide, NATA) of the different GLTP variants at 20 °C. Values are averages of at least 3 measurements

	Emission maxima (nm)	Quantum Yield
GLTP WT	347.8	0.028±0.0025
GLTP W85	350.6	0.021±0.0053
GLTP W96	345.0	0.052±0.011
GLTP W142	349.2	0.024±0.0074

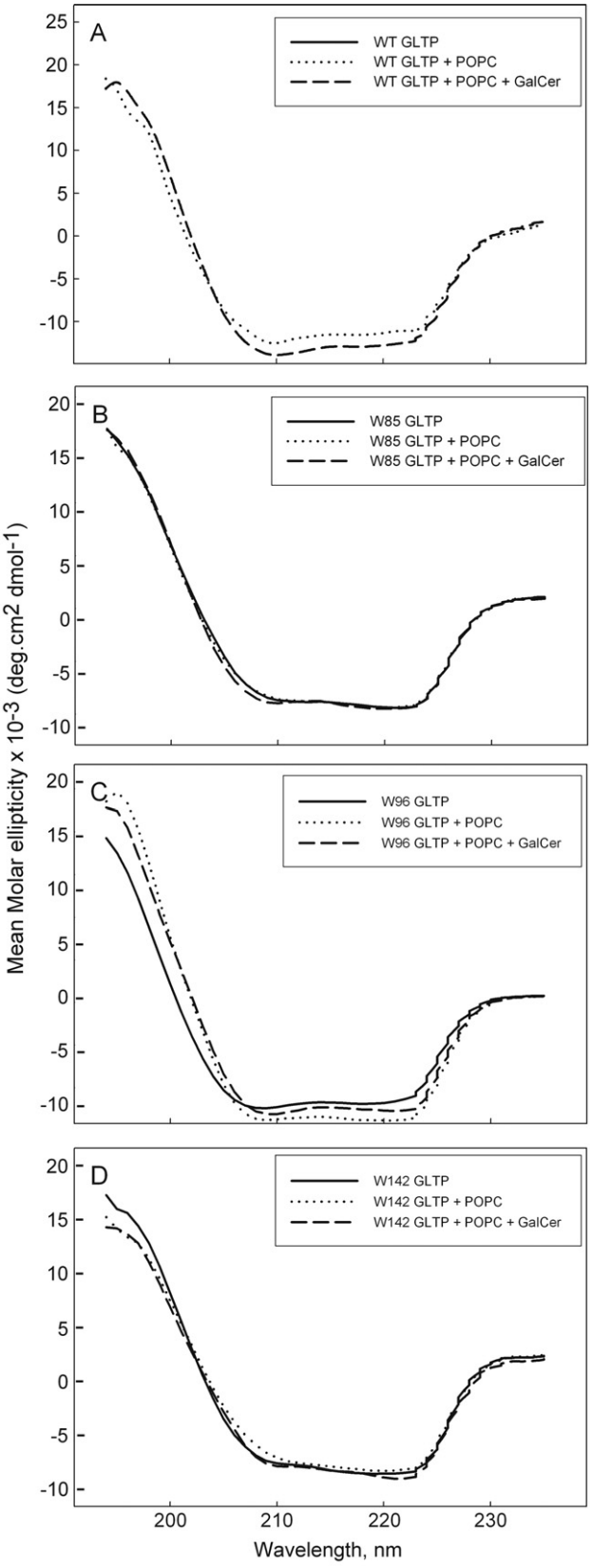

Fig. 4. Far UV circular dichroism spectra (190–240 nm) of wild-type GLTP and single tryptophan mutants (solid line), in the presence of lipid vesicles consisting of POPC (dotted line) and POPC vesicles containing 1 mol% galactosylceramide (dashed line). (A) Wild-type GLTP, (B) W85 GLTP, (C) W96 GLTP and (D) W142 GLTP. Protein concentration was 9.5 μM and lipid concentration 300 μM. Each spectrum represents the averages of 10 scans at 0.5 nm resolution at 37 °C using a quartz cuvette with a 0.1 mm path length.

2.7. Time-resolved measurements

By analyzing the tryptophan lifetime and anisotropy decay of wild-type GLTP and the three single tryptophan GLTP mutants it is possible to establish the mobility of the tryptophan residues. Tryptophan fluorescence decays were recorded and the decay was described with a one-exponential model since attempts to fit data to a two-exponential model did not give satisfying values of the goodness of fit χ^2 and Durbin–Watson parameters. One-exponential model fits gave a χ^2 value that was between 0.9 and 1.3, and the Durbin–Watson value between 1.5 and 1.7. The lifetimes of wild-type GLTP and the mutants are displayed in Table 3. NATA was measured as a standard and, was in our setup in good agreement with that reported earlier for NATA in water (3.17 ns) [22]. The tryptophan lifetime was 3.89 ns for wild-type GLTP, and no significant change occurred upon vesicle addition although POPC vesicles containing GalCer gave a slight decrease to 3.47 ns. The mutants W85 GLTP and W142 GLTP showed a lifetime of 2.88 ns and 2.96 ns, respectively, agreeing well with being in a polar environment.

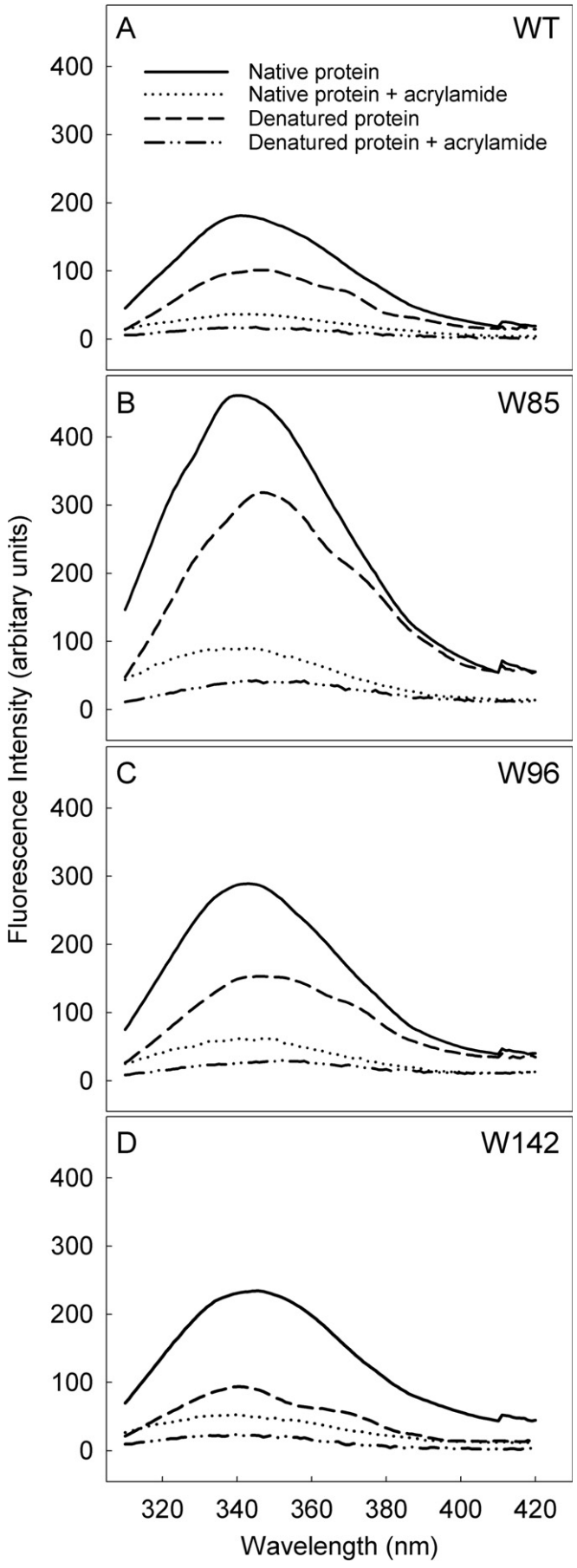

Fig. 5. Fluorescence emission of wild-type GLTP and single tryptophan mutants of native and denatured protein with or without acrylamide (0.55 M). The proteins were denatured with 6 M guanidine hydrochloride for 2 h at 25 °C. (A) Wild-type GLTP, (B) W85 GLTP, (C) W96 GLTP and (D) W142 GLTP. The protein concentration was 4.2 μM.

W96 GLTP had a tryptophan lifetime of 4.56 ns, indicting a more nonpolar environment. No change occurred when vesicles were added to mutants W85 GLTP and W142 GLTP. When vesicles were added to W96 GLTP the lifetime decreased for both types of POPC vesicles with or without GalCer. This suggests that the environment around tryptophan 96 became more polar after addition of vesicles.

Anisotropy decay measurements were performed to determine the rotational freedom of wild-type GLTP and the different mutants. Brownian rotation rates and the extent of motion are measured from the relative loss of parallel and perpendicular intensity during emission decays as described in Materials and methods. Anisotropy is dominated by rotation of tryptophan and segmental mobility in a nanosecond time scale [22]. The measured values are shown in Table 4. The slowest rotational correlation time of the single tryptophan mutants was seen with W142 GLTP, followed by W85 GLTP and W96 GLTP. The value of wild-type GLTP was much higher than the average of the mutants, probably indicating energy transfer or minor structural changes that were not detectable by our circular dichroism measurements (Fig. 4). The fast rotational correlation times demonstrate that the tryptophan is not immobilized but rather mobile, and that the measured times are not representing the protein correlation time which would be around 8–10 ns, typical of a protein tumbling in solution with the molecular size of GLTP [22].

3. Discussion

The transfer activity of wild-type GLTP and the only active mutant W96 GLTP was sensitive to changes in the pH of the transfer buffer. This is probably related to changes in the protein charge at different pH, since at neutral pH GLTP is slightly positively charged (isoelectric point ~9) while the zwitterionic phospholipid membrane is neutral. The membrane hydration changes caused by increasing salt concentrations are also likely to affect the GLTP activity. Changes in the pH affects the phospholipid membrane charge, as well as the charge of GLTP affecting the on- and off-rate of the protein from the membrane interface [19]. The minimal effect of ionic strength decrease could probably also be described to the contribution of charge changes both on the membrane and the protein, but clearly the protein is active even in low ionic strengths. It has recently been suggested that the rate limiting step in the GLTP mediated transfer of glycolipids would be the formation of a GLTP–glycolipid complex at the membrane interface, rather then the partitioning of GLTP into the lipid–water interface [25,26].

In the recent study of the human GLTP it was shown that when W96 was replaced by phenylalanine the activity was reduced to about 63% [3]. A replacement of W96 with alanine resulted in a completely inactive protein. It was also suggested

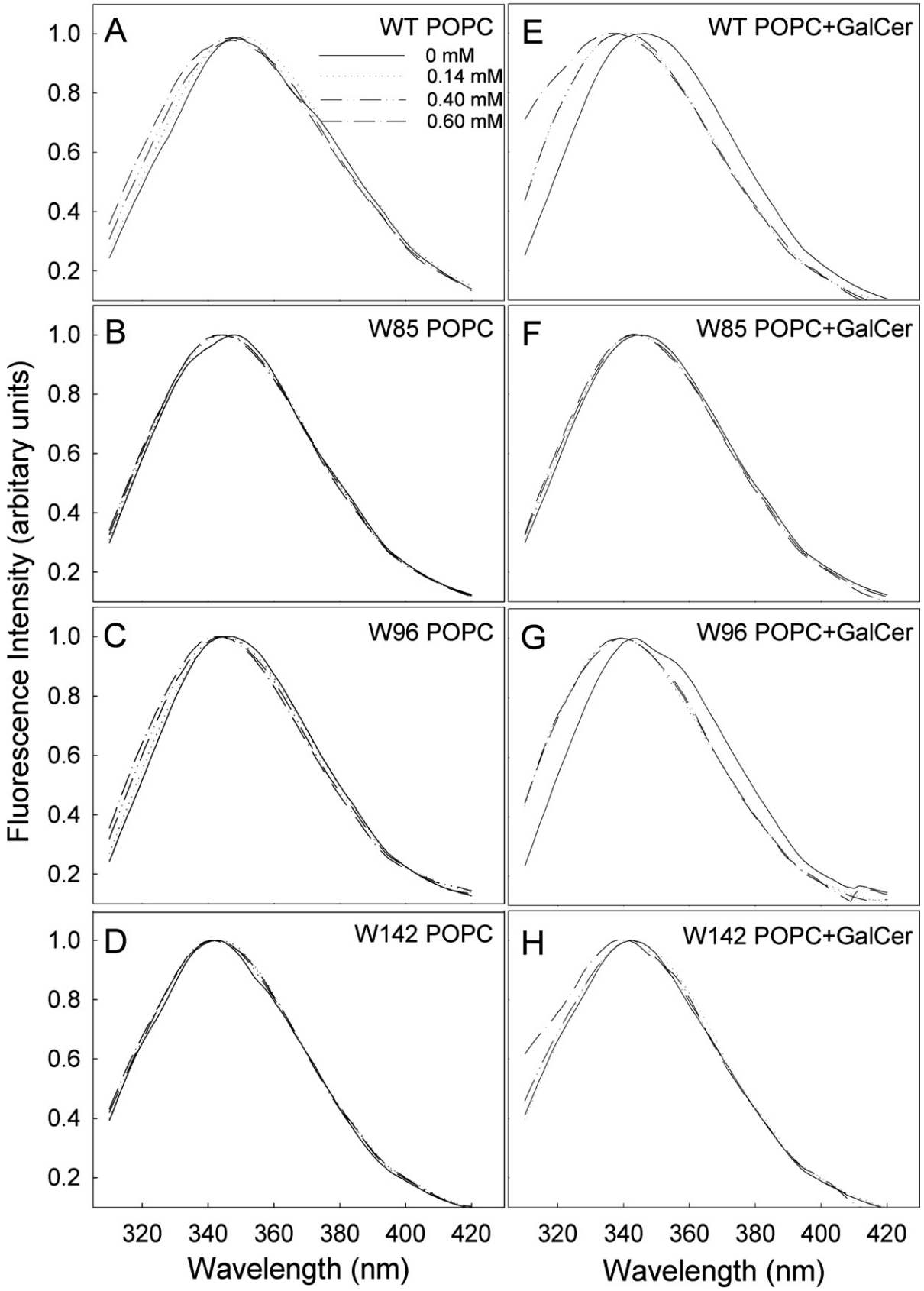

Fig. 6. Normalized fluorescence emission of wild-type GLTP and single tryptophan mutant with or without addition of vesicles. (A) and (E) Wild-type GLTP, (B) and (F) W85 GLTP, (C) and (G) W96 GLTP and (D) and (H) W142 GLTP. (A) through (D) with the addition of POPC vesicles (0 mM–0.60 mM), (E) through (H) with the addition of POPC vesicles containing 10 mol% GalCer (0 mM–0.60 mM). The protein concentration was 4.2 μM.

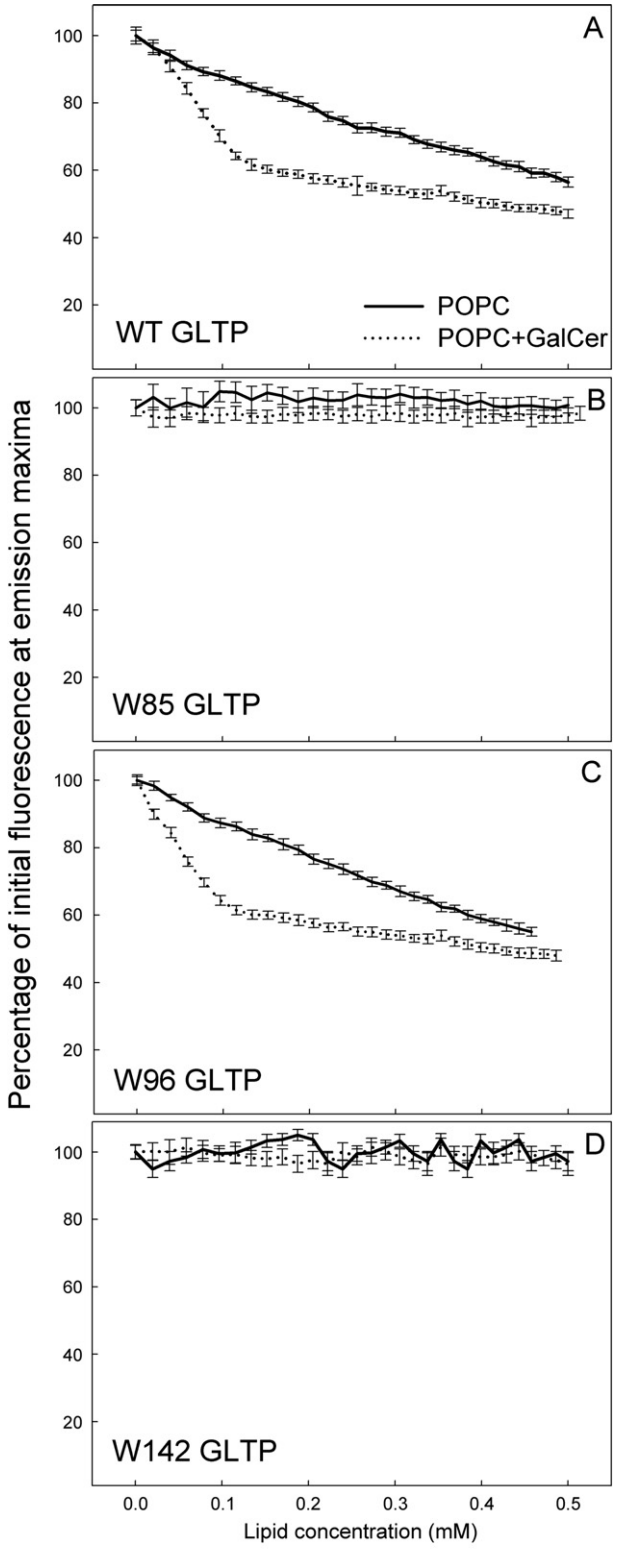

Fig. 7. Fluorescence titration curves of wild-type GLTP and single tryptophan mutants with lipid vesicles consisting of POPC (filled circles) and 10 mol% GalCer in POPC (open circles). (A) wild-type GLTP, (B) W85 GLTP, (C) W96 GLTP and (D) W142 GLTP. The intensity at the tryptophan emission maxima was plotted as a function of lipid concentration. A decrease in the intensity is an effect of tryptophan emission blue-shift (see Fig. 6). Protein concentration was 4.2 μM. The excitation wavelength was 295 nm. Values are averages of at least 3 measurements.

Table 2
Stern–Volmer quenching constant (K_{SV}) and fraction (f_a) of tryptophan fluorescence quenched by acrylamide at 37 °C

	K_{SV}, native	K_{SV}, denatured	f_a
GLTP WT	7.93	14.75	0.69
GLTP W85	15.64	18.60	0.97
GLTP W96	7.70	11.66	0.86
GLTP W142	3.63	6.37	1.00

Linear Stern–Volmer plot analyses were used to determine K_{SV} and modified Stern–Volmer plots to determine f_a values.

that W142 would participate in the GLTP membrane association, and that W96 is involved in recognizing the ligand by stacking over the glucose ring [3]. In this study we therefore intended to get better insight into the interaction of GLTP with membranes, especially the role of the tryptophan residues in the membrane interaction. With site-directed mutagenesis we constructed single tryptophan mutants in which tryptophan was replaced with phenylalanine. Only one mutant was active, W96 GLTP with W96 preserved and W85 and W142 mutated to phenylalanine, showing a 72% activity compared to wild-type GLTP. If W85 was preserved and W96 and W142 mutated to phenylalanine, or if W142 was preserved and W85 and W96 mutated to phenylalanine, the activity was completely lost.

The previous analysis of the hydrophobicity of the GLTP amino acid sequence by the Kyte–Doolittle method reveal four hydrophobic regions [1]. Three of theses centred at 36C, 99R and 189A are involved in the formation of the hydrophobic cavity, and the fourth cantered at 148F could potentially be involved in the protein membrane interaction. A semi-quantitative Wimley–White analysis [27] of the amino acid residues that would have a potential affinity for membrane interfaces also points to the sequence around 148F as being most favourable cluster. Replacing the residues in this region with a non-aromatic amino acid will probably be of interest to pursue in future studies.

Table 3
Tryptophan fluorescence lifetimes (τ, nm) at 350 nm of wild-type GLTP and single tryptophan GLTP mutants in the absence and presence of POPC or POPC/GalCer (99:1) vesicles

	Lifetime (ns) τ	χ^2	Durbin–Watson
WT GLTP	3.89±0.27	1.14±0.11	1.50±0.17
WT GLTP+POPC	3.94±0.14	1.14±0.19	1.58±0.06
WT GLTP+POPC+GalCer	3.47±0.44	0.97±0.21	1.74±0.30
W85 GLTP	2.88±0.10	0.93±0.14	1.71±0.24
W85 GLTP+POPC	3.12±0.03	1.00±0.05	1.86±0.42
W85 GLTP+POPC+GalCer	2.99±0.02	0.87±0.13	1.85±0.11
W96 GLTP	4.56±0.80	1.09±0.08	1.51±0.23
W96 GLTP+POPC	4.21±0.65	1.23±0.18	1.68±0.15
W96 GLTP+POPC+GalCer	3.49±0.05	0.89±0.21	1.80±0.40
W142 GLTP	2.96±0.20	1.18±0.07	1.54±0.36
W142 GLTP+POPC	2.80±0.03	1.03±0.13	1.60±0.09
W142 GLTP+POPC+GalCer	2.91±0.04	0.92±0.19	1.61±0.28

The measurements were performed at 37 °C. For further experimental details and analysis procedure, see Materials and methods. χ^2 and Durbin–Watson parameters are statistical goodness of fit parameters for the anisotropy decay fit. Values are averages of at least 3 measurements.

Table 4
Tryptophan rotational correlation times for wild-type GLTP and single tryptophan mutants

	Anisotropy decay (ns) ϕ
WT GLTP	0.233
W85 GLTP	0.159
W96 GLTP	0.183
W142 GLTP	0.125

Here we show that the active W96 GLTP mutant show a small blue shift in the emission in the presence of membranes containing no glycolipid, suggesting that the protein would come in contact with the surface of these membranes and is scooting for glycolipids, although positions 85 and 142 have phenylalanine instead of tryptophan. If the membrane contains glycolipids, an even greater blue shift was seen, probably because the glycolipid is bound to the GLTP in close vicinity to W96, and other environmental changes around W96. With the two mutants W85 GLTP and W142 GLTP that both have W96 replaced by phenylalanine we did not detect any blue shift in the tryptophan emission in the presence of POPC vesicles with or without GalCer. This suggests that the environment around W85 and W142 did not change much in the presence of membranes, probably because this region of the protein does not come close enough to the membrane interface. One should keep in mind that the mutant with both W85 and W142 intact but with phenylalanine at position 96 still showed an activity of 63% compared to wild-type GLTP [3]. Therefore it is likely that the W85 and W142 mutants are still scooting for glycolipids in the membrane. An introduction of a second mutation to either W85 or W142 would lead to an inability of the protein to adsorb to the membrane interface and bind and transfer glycolipids. We therefore conclude that W96, as well as either W85 or W142 is crucial for a fully active protein.

The circular dichroism measurements did confirm that the protein secondary structure is not affected by the substitution of tryptophan for phenylalanine. Neither did the shapes of the CD spectra change significantly due to addition of membranes, with or without glycolipids. A similar small difference in the ellipticity scale was also observed here for these mutants comparable to the earlier report [3]. Obviously CD measurements are not sufficient to detect small structural changes that could occur during GLTP lipid binding, and these questions will be addressed in further studies employing different NMR techniques.

The quantum yield of wild-type GLTP (0.028) was very close to the average for the mutants (0.032), indicating that no local perturbation occurred due to replacement of tryptophan with phenylalanine. The higher quantum yield for the W96 GLTP mutant would indicate that the tryptophan microenvironment was more non-polar than both environments around W85 and W142. Based on both lifetime and the quenching results it is also evident that the W96 is more shielded from the polar environment than W85 and W142.

The emission maximum for wild-type GLTP (348 nm) showed an average polar environment for the tryptophan residues. This result is in good agreement with the earlier reported values of 347 nm [7]. The X-ray structure reveals that W96 is located inside the binding cavity while both W85 and W142 are located at the entrance of the cavity [2]. The emission spectra support this since W85 showed the most polar environment followed by W142 and W96 being the most nonpolar. Denaturation of the samples produced an emission maximum closer to 350 nm which has been reported for NATA in water [24]. The Stern–Volmer constants showed a higher value for the denatured protein compared to the native, which was predicted [22]. Surprisingly, the K_{SV} of W142 suggests a nonpolar environment for the residue. One explanation to this could be that the residue has neighbouring nonpolar or charged amino residues. Electrostatic interactions and pH changes normally have big effects on a cationic protein [28]. This might be the reason why we could detect a difference in the sensitivity to pH for WT GLTP and W96 GLTP. The activity was more preserved at pH 10.0 than at pH 4.0, which implies that the protein is more active when it is positively charged. The difference between the pH and NaCl activity between wild-type GLTP and W96 GLTP is due to the lower over-all activity.

Both lifetime and anisotropy decay values show higher value for wild-type GLTP than for the measured average of the mutants. This suggests either quenching of tryptophan residues in the mutants or energy transfer in wild-type GLTP. As reported earlier amino acid residues known to quench tryptophan surround all three tryptophan [7]. Both mutants W85 GLTP and W142 GLTP show low lifetime values that is in agreement with exposed tryptophan residues. The lifetime of W96 GLTP decreased when vesicles were added indicating that the residue gets more exposed. This could be a consequence of the structural changes in the binding cavity and hydrogen bonding between W96 and the bound glycolipid, since W96 is directly involved in their binding [3].

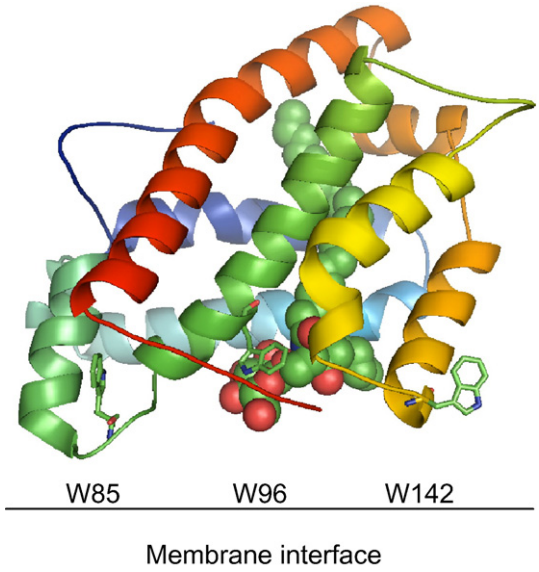

Fig. 8. Illustrative picture showing the suggestive GLTP (1TFJ) membrane interaction, with the location of the three tryptophan residues. The W96 environment is sensitive to bound glycolipid in the binding site cavity.

In Fig. 8 a model is presented indicating the involvement of W85 and W142 residues in the membrane interaction. GLTP does not seem to penetrate deeply into the membrane interfacial region when scooting for glycolipids [26], since we did not observe any changes in the W85 and W142 microenvironments in the presence of either POPC or POPC-GalCer vesicles. A complete transfer event of a glycolipid would however require tryptophan in positions 85 and 142 for a fully functional protein. We also show that GLTP does interact with vesicles containing only PC and does not require a glycolipid for interaction. This is well in agreement with recent findings based on RET measurements from GLTP to DPH and Laurdan [25].

Acknowledgments

We thank the team of Professor Arto Annila at University of Helsinki for access to the CD instrument, and Professor Julian G. Molotkovsky for the fluorescent AV-GalCer. Dr. Pia Roos-Mattjus and Heidi Kidron are acknowledged for valuable comments, and Elina Palonen for technical assistance.

This work was supported by the ISB Graduate School, Academy of Finland, Sigrid Jusélius Foundation, Magnus Ehrnrooth Foundation, Oscar Öflund Foundation, Svenska Kulturfonden, Medicinska understödsföreningen Liv och Hälsa r.f. and Åbo Akademi University.

References

[1] X. Lin, P. Mattjus, H.M. Pike, A.J. Windebank, R.E. Brown, Cloning and expression of glycolipid transfer protein from bovine and porcine brain, J. Biol. Chem. 275 (2000) 5104–5110.
[2] T.T. Airenne, H. Kidron, Y. Nymalm, M. Nylund, G. West, P. Mattjus, T.A. Salminen, Structural evidence for adaptive ligand binding of glycolipid transfer protein, J. Mol. Biol. 355 (2006) 224–236.
[3] L. Malinina, M.L. Malakhova, A. Teplov, R.E. Brown, D.J. Patel, Structural basis for glycosphingolipid transfer specificity, Nature 430 (2004) 1048–1053.
[4] P. Mattjus, B. Turcq, H.M. Pike, J.G. Molotkovsky, R.E. Brown, Glycolipid intermembrane transfer is accelerated by HET-C2, a filamentous fungus gene product involved in the cell–cell incompatibility response, Biochemistry 42 (2003) 535–542.
[5] P. Brodersen, M. Petersen, H.M. Pike, B. Olszak, S. Skov, N. Odum, L.B. Jorgensen, R.E. Brown, J. Mundy, Knockout of Arabidopsis accelerated-cell-death 11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense, Genes Dev. 16 (2002) 490–502.
[6] A. Godi, A. Di Campli, A. Konstantakopoulos, G. Di Tullio, D.R. Alessi, G.S. Kular, T. Daniele, P. Marra, J.M. Lucocq, M.A. De Matteis, FAPPs control Golgi-to-cell-surface membrane traffic by binding to ARF and PtdIns(4)P, Nat. Cell Biol. 6 (2004) 393–404.
[7] X.M. Li, M.L. Malakhova, X. Lin, H.M. Pike, T. Chung, J.G. Molotkovsky, R.E. Brown, Human glycolipid transfer protein: probing conformation using fluorescence spectroscopy, Biochemistry 43 (2004) 10285–10294.
[8] G. West, Y. Nymalm, T.T. Airenne, H. Kidron, P. Mattjus, T.T. Salminen, Crystallization and X-ray analysis of bovine glycolipid transfer protein, Acta Crystallogr., D 60 (2004) 703–705.
[9] A.S. Ladokhin, S. Jayasinghe, S.H. White, How to measure and analyze tryptophan fluorescence in membranes properly, and why bother? Anal. Biochem. 285 (2000) 235–245.
[10] G.R. Bartlett, Phosphorus assay in column chromatography, J. Biol. Chem. 234 (1959) 466–468.
[11] J.G. Molotkovsky, I.I. Mikhalyov, A.B. Imbs, L.D. Bergelson, Synthesis and characterization of new fluorescent glycolipid probes. Molecular organisation of glycolipids in mixed-composition lipid bilayers, Chem. Phys. Lipids 58 (1991) 199–212.
[12] M. Nylund, P. Mattjus, Protein mediated glycolipid transfer is inhibited FROM sphingomyelin membranes but enhanced TO sphingomyelin containing raft like membranes, Biochim. Biophys. Acta 1669 (2005) 87–94.
[13] P. Mattjus, J.G. Molotkovsky, J.M. Smaby, R.E. Brown, A fluorescence resonance energy transfer approach for monitoring protein-mediated glycolipid transfer between vesicle membranes, Anal. Biochem. 268 (1999) 297–304.
[14] L. Saunders, J. Perrin, D. Gammack, Ultrasonic irradiation of some phospholipid sols, J. Pharm. Pharmacol. 14 (1962) 567–572.
[15] O. Stern, M. Volmer, The extinction period of fluorescence, Phys. Z. 20 (1919) 188–189.
[16] S.S. Lehrer, Solute perturbation of protein fluorescence. The quenching of the tryptophyl fluorescence of model compounds and of lysozyme by iodide ion, Biochemistry 10 (1971) 3254–3263.
[17] C.A. Parker, W.T. Rees, Corrections of fluorescence spectra and the measurement of fluorescence efficiency, Analyst 85 (1960) 587–600.
[18] P. Mattjus, A. Kline, H.M. Pike, J.G. Molotkovsky, R.E. Brown, Probing for preferential interactions among sphingolipids in bilayer vesicles using the glycolipid transfer protein, Biochemistry 41 (2002) 266–273.
[19] P. Mattjus, H.M. Pike, J.G. Molotkovsky, R.E. Brown, Charged membrane surfaces impede the protein mediated transfer of glycosphingolipids between phospholipid bilayers, Biochemistry 39 (2000) 1067–1075.
[20] N. Sreerama, R.W. Woody, On the analysis of membrane protein circular dichroism spectra, Protein Sci. 13 (2004) 100–112.
[21] N. Sreerama, R.W. Woody, Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set, Anal. Biochem. 287 (2000) 252–260.
[22] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Principles of Fluorescence Spectroscopy, Kluwer Academic/Plenum, New York, 1999, pp. 1–698.
[23] M.R. Eftink, C.A. Ghiron, Exposure of tryptophanyl residues in proteins. Quantitative determination by fluorescence quenching studies, Biochemistry 15 (1976) 672–680.
[24] A.R. Merrill, L.R. Palmer, A.G. Szabo, Acrylamide quenching of the intrinsic fluorescence of tryptophan residues genetically engineered into the soluble colicin E1 channel peptide. Structural characterization of the insertion-competent state, Biochemistry 32 (1993) 6974–6981.
[25] C.S. Rao, T. Chung, H.M. Pike, R.E. Brown, Glycolipid transfer protein interaction with bilayer vesicles: modulation by changing lipid composition, Biophys. J. 89 (2005) 4017–4028.
[26] C.S. Rao, X. Lin, H.M. Pike, J.G. Molotkovsky, R.E. Brown, Glycolipid transfer protein mediated transfer of glycosphingolipids between membranes: a model for action based on kinetic and thermodynamic analyses, Biochemistry 43 (2004) 13805–13815.
[27] W.C. Wimley, S.H. White, Experimentally determined hydrophobicity scale for proteins at membrane interfaces, Nat. Struct. Biol. 3 (1996) 842–848.
[28] M. Rytömaa, P.K.J. Kinnunen, Evidence for two distinct acidic phospholipid-binding sites in cytochrome *c*, J. Biol. Chem. 269 (1994) 1770–1774.